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14. ABSTRACT With the support from this grant we have been developing photocross-linking DNA base analogues to trap and identify DNMTs and interacting proteins on specific promoter DNA. To this end Diazirine photophore was introduced into either the major or minor groove of DNA as a photocross-linking group via a convertible nucleoside methodology. The resulting DNA probes efficiently cross-linked with the E. coli DNA adenine methyltransferase (EcoDam). The diazirine photophore possesses clear advantages over other photocross-linking groups previously installed on DNA in that it has the least steric hindrance and efficiently generates a carbene intermediate that non-specifically cross-links with protein residues nearby in high efficiency. We have also applied the method to human cell extracts and obtained very promising results. Based on this early success we are working on the BRCA1 promoter DNA and hope to identify the DNMT that works on this promoter in the near future.					
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Introduction

Hypermethylation of several gene promoters contribute to the growth of breast cancer cells. Despite extensive efforts to understand the cause of the abnormal DNA methylation in breast cancer, little is understood about the factors that contribute to hypermethylation. We have been developing unique chemical tools that would allow us to covalently trap DNA methyltransferases (DNMTs) or proteins associated with DNMTs to specific promoter DNA. With this method we can systematically identify DNMTs that perform methylation function on various promoter regions. We can also identify proteins that may direct this methylation to specific regions of a genome. In the last year we have spent most time developing the photocross-linking chemistry and testing the concept. The idea starts to work and we are currently working on human cells including breast cancer cells

Body

The summary will be divided into two parts.

1. Developing diazirine-based DNA photocross-linking probes for studying protein-DNA interactions. This work has been written up and submitted to *Angew. Chem.*¹ The manuscript is attached in the Appendices. Please see details there.

2. Using the cross-linking chemistry to study DNMTs in human cells. In collaboration with Dr. Lucy Godley in the University of Chicago Hospital we prepared and synthesized several DNA probes and tested trapping DNMT and proteins associated with DNMTs. Briefly, 293 cells were pelleted and lysed in Non-Denaturing Cellular Lysis Buffer (20mM Tris-HCl pH 8.0, 50mM NaCl, 1mM EDTA, 0.5% NP40) to make a cytoplasmic extract. The nuclei were pelleted and lysed in Non-Denaturing Nuclear Lysis Buffer (20mM Tris-HCl pH 8.0, 250mM NaCl, 1mM EDTA, 0.7% NP40). Between 30-50µg of nuclear protein extract were combined with the modified DNA oligonucleotide (1 nmol) and incubated at 4 °C for 16 h. After incubation, the samples were irradiated with a mercury vapor lamp (Ace Glass Incorporated, Vineland, New Jersey) for 10 minutes. This lamp uses a 450 W power supply, and light below 300 nm was cut off using a filter. The protein-DNA complexes were immobilized using streptavidin beads via the biotin tags, washed gently, and the proteins eluted with high salts or under denaturing conditions. The eluted proteins were analyzed Western blotting for the biotin group and antibodies directed against different DNMTs. DNMTs that have been cross-linked to the probe will show an apparent shift in molecular weight. Other proteins associated with DNMTs would show up in the biotin western. One example of our results is shown in Figure 1. When we use immunoprecipitated complex with DNMT3b antibody and treated and irradiated with our DNA probe, we observed two proteins, xxx and yyy, associated with the complex. Our preliminary

data also suggested DNMTs that were trapped down. We need to vary DNA length and optimize the probe for this assay, which we are performing.

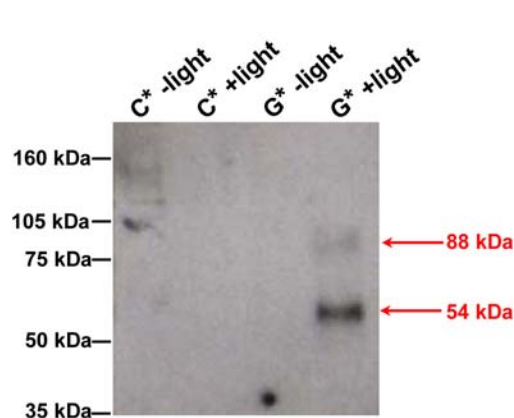


Figure 1. Non-denaturing nuclear protein extracts were made from 293 (human embryonic kidney) cells. An immunoprecipitation for DNMT3B was performed using the T-16 anti-DNMT3B antibody (Santa Cruz Biotechnology, Inc.). The immunoprecipitates were incubated with biotinylated oligonucleotides corresponding to a unique sequences of human chromosome 8, just 5' to a LINE1 element. The oligonucleotides contain a modified base to allow protein cross-linking, either a diazirine-modified cytosine (C*), which projects the derivatized base into the major groove, or a diazirine-modified guanine (G*), which projects the derivatized base into the minor groove. The protein extracts were incubated with the oligonucleotides for 16 hours at 40C. Half of each sample was treated with ultraviolet light, and the immunoprecipitates were washed extensively. The components of the immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose, and a Western blot for biotin was performed. Two cross-linked proteins (indicated in red to the right of the figure) were identified using the oligonucleotide with the derivatized guanine base and ultraviolet treatment to activate the diazirine group. These proteins had apparent molecular weights of 88kDa and 54 kDa, respectively. The positions of protein molecular weight standards are given to the left.

Key Research Accomplishments

We have developed a series of photo-active cross-linking DNA base analogues that worked in cell extracts. These probes will offer tools to study DNA methylation in our and other groups in the future.

Reportable Outcomes

A manuscript was submitted to *Angew. Chem.* The PI has given a lecture on related research: W. M. Keck Foundation, April 24, “*Study Protein-DNA Interactions*”; two grants applications to NIH and March of Dime foundation were sent out based on preliminary results obtained from this work.

Conclusion

The support of this grant is essential for my group to initiate this new research direction. Now I have one student focusing on developing these DNA-based probes and one senior research staff working on the cell biology part of the project. In the next few years I expect major discoveries coming out of this work.

References

1. Shigdel, U. K., Zhang, J. and He, C. “Diazirine–Based DNA Photocross–Linking Probes for Studying Protein–DNA Interactions” Submitted.

Appendices

Shigdel, U. K., Zhang, J. and He, C. “Diazirine–Based DNA Photocross–Linking Probes for Studying Protein–DNA Interactions” Submitted.

Diazirine-Based DNA Photocross-Linking Probes for Studying Protein–DNA Interactions

Uddhav K. Shigdel, Junliang Zhang, and Chuan He*

Protein–DNA interactions occur in fundamental life processes such as replication, transcription, DNA modification and DNA repair. Chemical and photochemical cross-linking have been extensively used in probing protein–DNA interactions.^[1,2,3] Chemical cross-linking methods enable trapping and characterizing protein–DNA complexes in various forms that are labile in the absence of covalent linkages.^[2] Photocross-linking has been widely utilized to map out protein residues involved in protein–DNA interactions and trap/identify proteins that interact with DNA.^[1,3]

Photocross-linkers providing high yields of cross-linking are essential for obtaining enough materials for characterization. Various photoactive groups have been introduced to DNA for cross-linking studies;^[1,3] however, most of these probes suffer from the low cross-linking efficiency either due to low reactivity or photo-decomposition, which makes biochemical studies tedious and sometimes unreliable. Aryl azides and benzophenone can be tethered to DNA or RNA via thiol-modified DNA or RNA backbones. Such probe has been utilized to obtain information on protein–DNA and protein–RNA interactions.^[3c,d] However, the size of these groups may interfere with bindings that are sensitive to steric hindrance.

We envision installing the diazirine photophore into DNA as a photocross-linking group. Under UV irradiation diazirine eliminates a N₂ unit and forms a reactive carbene intermediate (Figure 1).^[1,5] This carbene species can readily cross-link with nearby protein residues in high efficiency. The use of diazirine has several advantages: (i) the photo-generated carbene intermediate has high photocross-linking efficiency; (ii) diazirine has superb chemical stability prior to photolysis and it photolyzes rapidly at wavelengths beyond the UV absorbance of most biological macromolecules; iii) introduction of small sized diazirine reduces potential steric hindrance; iv) moreover, this method is non-specific and does not require presence of specific protein residues for cross-linking. Trifluoromethyl-aryl-diazirine has been installed on DNA bases,^[4] however the large size of the modification still imposes the steric problem we wish to address.

Our strategy is to use the convertible nucleoside method^[6] to introduce diazirine into the major and minor grooves of DNA. Diazirine amines with two- and three-carbon linkers (C2, *n* = 1; C3, *n* = 2, Figure 1) were synthesized by modifying a reported

procedure.^[5] These diazirine amines were incorporated into the major and minor grooves of DNA via the convertible nucleoside methodology (Figure 1).^[6]

Specifically, oligonucleotides were prepared from solid-phase syntheses with O⁴-triazolyl-dU-CE phosphoramidite or 2-F-dI-CE phosphoramidite incorporated at specific positions. Diazirine amines were introduced via postsynthetic modification/deprotection as shown in Figure 1.^[6]

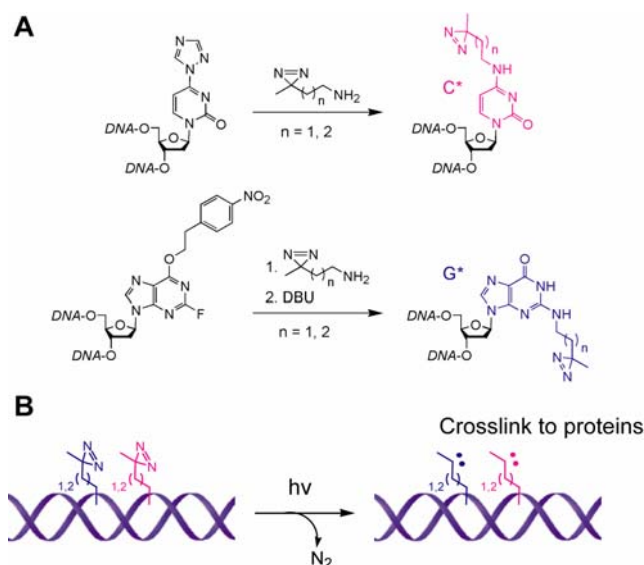


Figure 1. Incorporation of diazirine into the major and minor grooves of DNA. (A) Installing diazirine with C2 (*n* = 1) or C3 (*n* = 2) linker into the major (by deprotecting O⁴-triazolyl-dU-substituted DNA) or the minor groove (by deprotecting 2-F-dI-substituted DNA) of DNA by using the corresponding diazirine amines. (B) The diazirine-modified DNA can be photoactivated to generate a carbene intermediate for cross-linking to DNA-binding proteins.

Single-stranded DNA 1 and 6 were made with C* or G* (Figure 2). C* stands for a N⁴-modified cytosine while G* indicates a modification at the N²-position of guanosine. Annealing ssDNA-1 with complementary strands gave double-stranded DNA probes 2, 3 and 4 with G, A or T opposite to the diazirine-modified C*, respectively (Figure 2).

Annealing a complementary strand with an abasic site opposite to the C* gave dsDNA-5. DNA probes 7–15 were prepared with the minor group modified G*. Annealing complementary strands to ssDNA-6 yielded DNA-7 and DNA-8 with C or A opposite to G*, respectively (Figure 2). DNA probes 9–11 were prepared after annealing ssDNA-6 to complementary strands containing an abasic site. DNA probes 12–15 were prepared with G* positioned in matched or mismatched base pairs adjacent to an abasic site (Figure 2).

In order to determine if the diazirine-tethered duplex DNA is any different from the analogous alkylamine- or disulfide-tethered dsDNA, we measured the melting temperatures (*T*_m) of the modified duplex oligonucleotides by using the Differential Scanning Calorimeter (DSC).^[10] The *T*_m values of dsDNA-2 and dsDNA-7 with C2 linker were determined to be 55.0 °C and 61.2 °C, respectively (Table 1). The differences in *T*_m values between the diazirine-tethered DNA and normal DNA correlate with the differences of that for alkylamine- or disulfide-tethered dsDNA

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


and normal dsDNA obtained previously.^[6b,d] Thus, the diazine tether does not introduce extra destabilization than simple alkylamines as expected.

Since carbene can indiscriminately cross-link to organic groups nearby, we further tested if carbene generated from diazine will give any interstrand cross-linking in dsDNA. Among various major and minor groove probes that we tested, we did not observe any interstrand cross-linking products (data not shown).



Figure 2. DNA sequences used in photocross-linking studies.

Table 1. Melting Temperatures (T_m) of Normal and Modified Oligonucleotides.

Oligonucleotide	Melting Temperature ($^{\circ}\text{C}$)
5'-ATCAAAGCGCAAGAC-3' 3'-TAGTTTCGCGTTCTG-5' 	60.6
dsDNA-2 5'-ATCAAAGCGCAAGAC-3' 3'-TAGTTTCGCGTTCTG-5' 	55.0
dsDNA-7 5'-ATCAAAGCGCAAGAC-3' 3'-TAGTTTCGCGTTCTG-5' 	61.2

To showcase the utility of these DNA probes we performed photocross-linking of these probes with DNA-binding proteins. We chose *E. coli* DNA adenine methyltransferase (EcoDam) as the first example. EcoDam methylates the N6 position of adenine in GATC sequence. Structural information on how this protein interacts with the sequence-specific DNA was limited until a recent structural report.^[7a] We were interested in how this protein interacts with DNA, in particular, its sequence non-specific DNA binding mode.

We expressed and purified EcoDam and studied its photocross-linking to various DNA probes containing diazine in either the major or the minor groove of duplex DNA. We mixed 1 equiv of EcoDam with 3 equiv of various DNA probes on ice for 16 h before irradiating with a mercury lamp for 10 min. Samples were

then analyzed by Coomassie Blue stained SDS-PAGE gel. Appearance of a low mobility band indicated formation of cross-linked products. To our delight, very good yields (20-50%) of cross-linking were obtained with most of the DNA probes (notably with probes 1, 2, 3, 6, 10 and 11, Figure 3). To ensure maximum cross-linking, all experiments were performed with 3 equiv of DNA and 1 equiv of EcoDam. However, the use of excess equivalents of DNA probes may not be necessary (Figure 3A, compare lanes 2, 4 and 7). Ten min seems to be an optimal UV irradiation time to initiate cross-linking (Figure 3A, compare lanes 6, 7 and 8).

To test the versatility of these probes, we surveyed a range of different probes shown in Figure 2. In the first group of probes tested, we positioned the diazine-containing base opposite to an abasic site or in a matched or mismatched base pair. We found that probes 2, 3, 10 and 11 gave the best results with ~40-50% cross-linking yields (Figure 3A, lane 7; Figure 3C, lane 6; Figure 3D, lanes 10, 12, 14 and 16). If the reaction mixture was preincubated with 10-fold excess of unmodified dsDNA, no significant cross-linking could be observed between EcoDam and one of the best probes, dsDNA-10 (Figure 3A, lane 10). This DNA probe does not contain the sequence recognized by EcoDam. It can be titrated away with excess of unmodified DNA, further demonstrating that protein-DNA interaction is critical for photocross-linking.

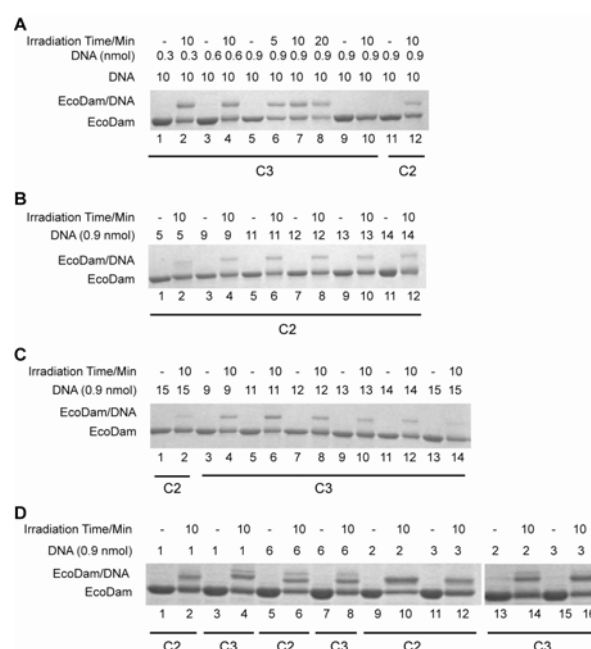


Figure 3. SDS-PAGE analysis of the photocross-linking reactions between *E. coli* DNA adenine methyltransferases (EcoDam, 0.3 nmol) and various DNA probes (0.9 nmol). C2 and C3 are carbon tether lengths between the diazine ring and guanosine N2 or cytosine N4. DNA probes 2, 3, 10 and 11 gave the best cross-linking yield.

Next, we tested probes that contain G* adjacent to the abasic site (probes 12-15, Figure 2). We picked G* since the minor groove modification gave the best cross-linking yield among DNA probes that contain an abasic site. We saw moderate cross-linking of EcoDam with probes 12, 13 and 14 with both C2 and C3 linkers, whereas minimal cross-linking was observed when probe 15 was used (Figures 3B and 3C). The minor groove modification on probes 7 and 8 could also form cross-linked products with EcoDam (not as efficient as probes 1, 2, 3, 6, 10 and 11, data not shown), but probes 10 and 11 gave the best yields (~50%). Base flipping proteins such as EcoDam are known to recognize unstable sites in a specific manner,^[8] which may account for the higher yields of cross-linking observed for probes 10 and 11. When single-stranded DNA probes 1 and 6 were used, in addition to 1:1 protein-DNA complexes, we also observed 1:2 protein-DNA complexes on the gel (Figure 3D).

It might be due to a small fraction of EcoDam that binds to two ssDNA simultaneously. These results showed that one can identify several DNA probes that efficiently cross-links with the target protein from a quick screen (Table 2).

Considering that a non-specific sequence was used in the study of EcoDam, experiments with probes 2 and 3 are excellent results for photocross-linking reactions between protein and DNA. The result suggests that these DNA probes were reacting with DNA binding residues on the surface of EcoDam because both matched and mismatched probes gave comparable photocross-linking yields. EcoDam methylates adenine in a sequence-specific manner.^[7] It must scan through DNA base pairs while searching for its specific sequence. The close contacts with DNA base pairs may account for cross-linking with diazirine on matched DNA probes. Since we can now trap this “non-specific” interaction in high yields, it is promising to structurally characterize this interaction which will give valuable information on how EcoDam binds to non-specific DNA.

Table 2. Summary of the Probes that Most Efficiently Photocross-Linked with EcoDam.

Oligonucleotide	Modification	Better Carbon-length
dsDNA-2		
5'-ATCAAAGGCAAGAC-3'	C	2
3'-TAGTTTCGCGTTCTG-5'		
dsDNA-3		
5'-ATCAAAGGCAAGAC-3'	C	3
3'-TAGTTTCACGTTCTG-5'		
dsDNA-10		
5'-ATCAAAGGCAAGAC-3'	G	3
3'-TAGTTTCGCGTTCTG-5'		
dsDNA-11		
5'-ATCAAAACGCAAGAC-3'	G	3
3'-TAGTTTCGCGTTCTG-5'		

We also picked human O⁶-alkylguanine-DNA alkyltransferase (hAGT) for study. This protein locates DNA base damage sequence non-specifically. It flips and inserts the damaged base into the active site, and irreversibly transfers the alkyl group to a nucleophilic Cys residue.^[9] When hAGT was incubated with our DNA probes, very good yields of cross-linked products (Figure 4A, lanes 5, 7, 9-11 and 13; Figure 4B, lanes 2, 4, 6, 8 and 10) were obtained. Experiments with the use of various ratios of dsDNA-3/hAGT indicated that 3 equiv of DNA could consume almost all hAGT in solution (Figure 4A, compare lanes 5, 7 and 10). Time dependence study showed that 10 min seems to be an optimal UV irradiation time (Figure 4A, compare lanes 9, 10 and 11).

Low cross-linking was observed when the DNA probe 2 was used with either C2 or C3 linker (Figure 4A, lane 3; Figure 4B, lane 8); whereas the mismatched probe 3, with either C2 or C3 linker, gave a very good yield (50-75%) of photocross-linked product (lane 10 in both Figures 4A and 4B). DNA probe 4, with C*:T mismatch, gave less cross-linking compared to probe 3 (C*:A) as shown in Figure 4B. Single-stranded DNA-1, with both C2 and C3 linkers showed little cross-linking (Figure 4B, lanes 2 and 4). Addition of 10-fold excess of unmodified, mismatched dsDNA into the reaction mixture did yield less covalently linked complex (Figure 4A, lane 13), indicating that hAGT can be titrated away from dsDNA-3 with mismatched dsDNA. We observed minimal photocross-linking of hAGT with dsDNA-5 and other DNA probes containing minor groove modifications (results not shown), most likely due to lack of contact from the tethered diazirine to the protein.^[9b,c] Overall, dsDNA-3 with C2 linker worked most efficiently with hAGT.

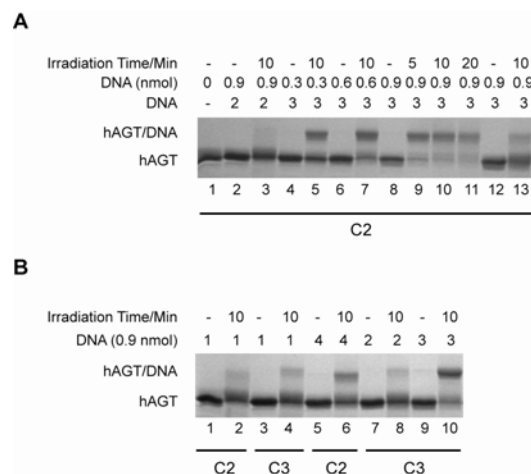


Figure 4. SDS-PAGE analysis of the photocross-linking reactions between human AGT (0.3 nmol) and DNA probes 1-4 (0.9 nmol). C2 and C3 are carbon tether lengths between the diazirine and the cytosine N4. dsDNA-2 gave minimal cross-linking, where as dsDNA-3 with C2 linker gave most efficient cross-linking.

In summary, we show here a simple and quick method to install diazirine into the major and minor grooves of DNA for efficient photocross-linking to proteins. In two examples we provided here, good to excellent cross-linking yields were obtained with DNA probes bearing small diazirine modifications. This method appears to be well suited for mapping out protein-DNA interactions, especially those that may be sensitive to steric hindrance. The high cross-linking efficiency observed in several cases would allow direct purification of the photocross-linked products for structural characterization. Alternatively, protein residues engaged in photocross-linking can be readily identified by mass spectrometry and mutated to Cys residue to set up for chemical disulfide cross-linking with disulfide-tethered oligonucleotides. The disulfide-linked complex can be subjected for structural studies. In addition, the relatively high cross-linking efficiency of these diazirine-based probes makes them promising tools for covalently trapping proteins from cell extracts.

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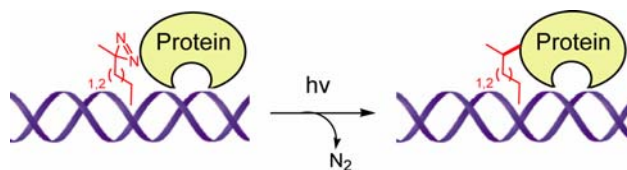
Keywords: convertible nucleoside method· photocross-linking· protein-DNA cross-linking· diazirine

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Diazirine-Based DNA Photocross-Linking Probes for Studying Protein-DNA Interactions



Diazirine can be installed in the major and minor grooves of DNA for efficient photocross-linking to proteins.

Supporting Information

Diazirine-Based DNA Photocross-Linking Probes for Studying Protein-DNA Interactions

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General: All reagents were obtained from commercial sources and used as received unless otherwise noted. Inert N₂ atmosphere was employed for all reactions and all solvents were distilled unless otherwise noted. Forced-flow chromatography on EM Science Geduran silica gel 60 (35-75 μ m) was used to purify products. Thin layer chromatography was performed on EM Science silica gel 60 F254 plates (250 μ m). Developed chromatogram was visualized by Ultra Violet lamp and/or by staining with aqueous potassium permanganate/K₂CO₃ solutions. Nuclear magnetic resonance (NMR) spectra for ¹H were acquired with CDCl₃ on Bruker DRX-500/400 operating at 500/400 MHz; TMS was used as internal standard for calibration purpose. Data for ¹H NMR are recorded as follows: chemical shift (ppm), multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet), integration, coupling constant (Hz). High-Resolution Mass was performed by Mass Spectrometry Facility at University of Norte Dame.

Experimental Procedure to Synthesize Diazirine Amines:

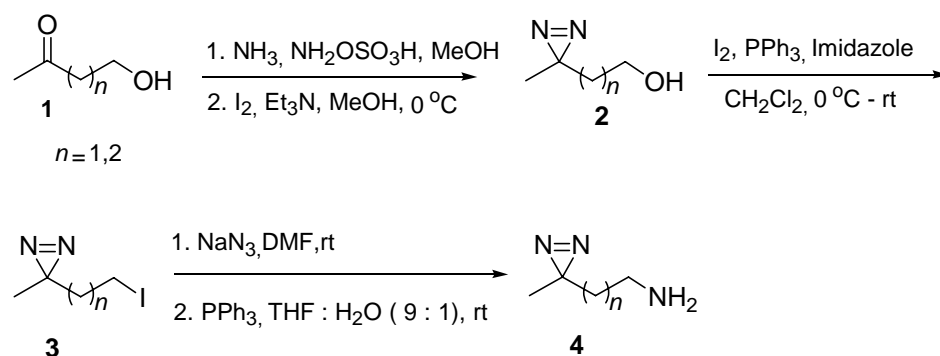


Figure S1. The synthetic route for making diazirine amines.

Two-carbon and three-carbon diazirine amine were made using similar procedure

2-(3-Methyl-3H-diazirin-3-yl)-ethanol (2, n=1)

4-hydroxy-2-butanone (45 g, 0.51 mol) was added to 200 mL of NH₃ and stirred for 3 h at -78 °C. Hydroxylamine O-Sulfonic acid (63.5 g, 0.56 mol) was dissolved in methanol and poured into the reaction mixture. After overnight stirring, the white precipitate was filtered and methanol was added to ice cold reaction mixture followed by triethylamine. Iodine was slowly added until the color of iodine persists. After 2 h methanol was evaporated and the reaction mixture was extracted with ether and dried over MgSO₄. Vacuum distillation was done to get the final pale yellow oil **2** (15 g, 30% overall yield).

3-(2-Iodo-ethyl)-3-methyl-3H-diazirine (3, n=1)

One equivalent of triphenylphosphine was dissolved in 50 mL of ice cold dichloromethane. After dissolving triphenylphosphine, iodine was added followed by imidazole. Once the color of iodine vanished compound **2** (6.06 g, 0.06 mol) was added and stirred overnight. The product was extracted with ether, washed with water and dried over MgSO₄. After solvent evaporation, the product was purified by column chromatography on silica gel (pure hexane) to give compound **3** (4.05 g, 33%).

2-(3-Methyl-3H-diazirin-3-yl)-ethylamine (4, n=1)

One equivalent of NaN₃ was added to **3** (4.05 g, 0.02 mol) and the reaction mixture was stirred at room temperature overnight. The product mixture was extracted with ether and after ether evaporation tetrahydrofuran:water (9:1) was added followed by triphenylphosphine. After 2 h 1N HCl was added to the product mixture and extracted with ether to remove the

byproducts. Subsequently 1N NaOH was added to the mixture and after ether extraction yielded the final product **4**. (1.6 g, 80% crude). $^1\text{H-NMR}$ (CDCl_3) * 2.57 (t, 2H, $J = 7.0$ Hz), 1.54 (t, 2H, $J = 7.0$ Hz), 1.04 (s, 3H).

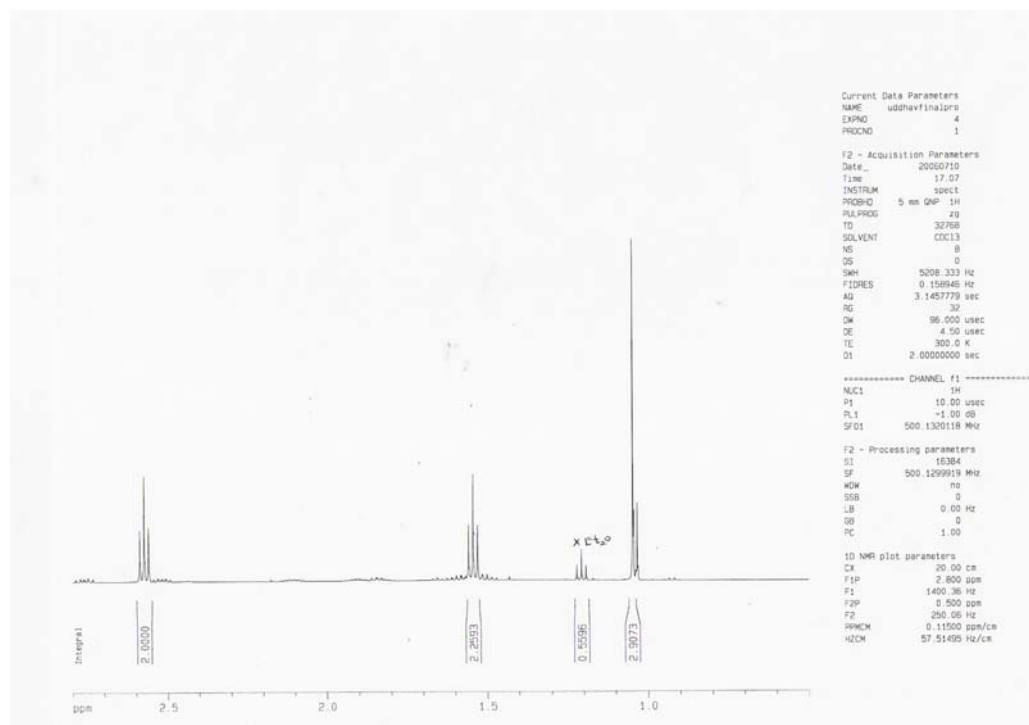


Figure S2. $^1\text{H-NMR}$ of 2-(3-Methyl-3H-diazirin-3-yl)-ethylamine (**4**, $n=1$).

3-(3-Methyl-3H-diazirin-3-yl)-propylamine (4**, $n=2$)** was made with 3-acetyl-1-propanol as a starting material following the same procedure as making (**4**, $n=1$).

$^1\text{H-NMR}$ (CDCl_3) * 2.66 (t, 2H, $J = 6.9$ Hz), 1.38 (m, 2H), 1.32 (m, 2H), 1.01 (s, 3H); FABMS m/z $[\text{MH}]^+$; calculated for $\text{C}_5\text{H}_{11}\text{N}_3$ 114.1031 found 114.1021.

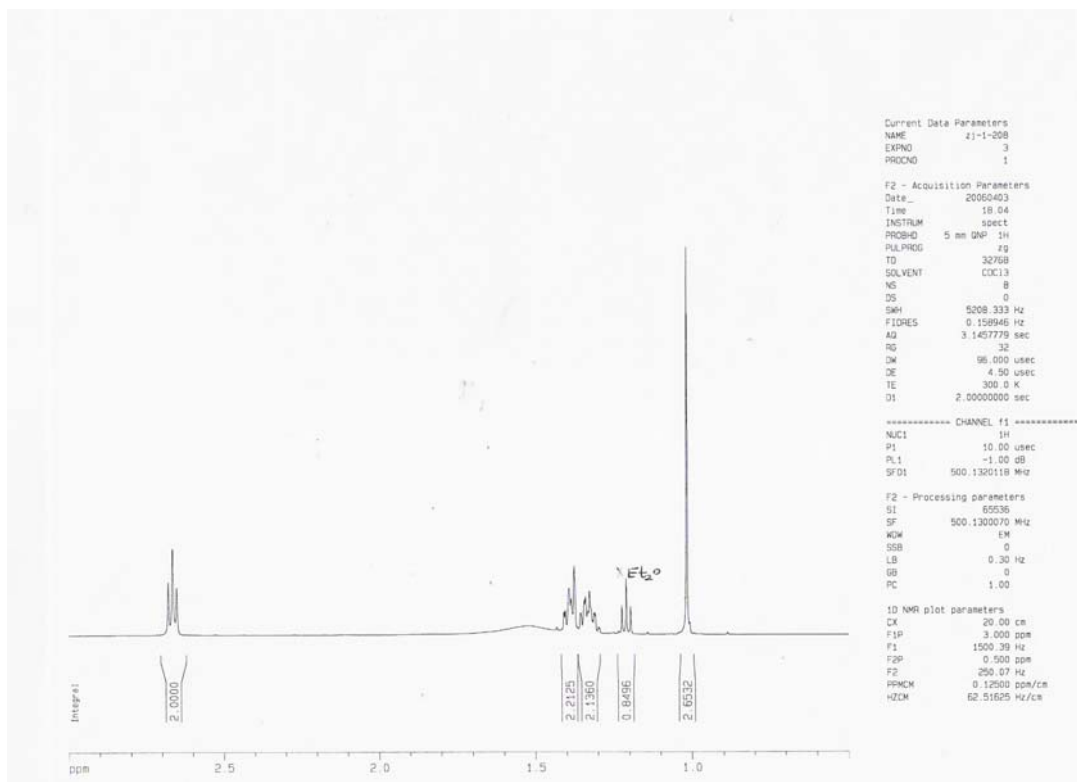


Figure S3. ^1H -NMR of 3-(3-Methyl-3H-diazirin-3-yl)-propylamine (**4**, $n=2$).

Expression and Purification of Human AGT. Human AGT was purified as described.^[2d]

Expression and Purification of DNA Adenine Methyltransferase. *E. coli* cells containing a construct of DNA adenine methyltransferase was grown aerobically with chloramphenicol (30 $\mu\text{g/mL}$) at 37 $^\circ\text{C}$ until the OD_{600} reached 0.7. IPTG (1 mM) was added and the cells were grown at 30 $^\circ\text{C}$ for 4 h. The cells were harvested by centrifugation and stored at -80 $^\circ\text{C}$. All subsequent steps were performed at 4 $^\circ\text{C}$. The cell pellet was resuspended in 20 mL of lysis buffer (10 mM Tris-HCl [pH 7.4], 300 mM NaCl, 5% glycerol, 2 mM CaCl_2 , 10 mM MgCl_2 , 10 mM 2-mercaptoethanol), disintegrated by sonication, and centrifuged at 12,000 rpm for 20 min. The supernatant was loaded onto HisTrap FF (GE Healthcare) that had been equilibrated with buffer A (10 mM Tris-HCl [pH 7.34], 150 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol), and was washed with 20% buffer B (10 mM Tris-HCl [pH 7.34], 150 mM NaCl, 400 mM imidazole, 10 mM 2-mercaptoethanol, 5% glycerol) and finally eluted with a linear gradient of imidazole (0-400 mM). Fractions containing the protein were concentrated by ultrafiltration (Centricon YM10 membrane; Amicon, Millipore Corporation, Bedford, MA) and purified further with a Mono-S cation exchange column (Amersham Biosciences) using a linear gradient of NaCl (0-1.0 M).

Oligonucleotide Synthesis. Oligodeoxynucleotides were synthesized on an Expedite Nucleic Acid Synthesizer from PE biosystems. Major groove modifications were made by incorporating O^4 -triazolyl-dU-CE phosphoramidite (Glen Research) at the modified positions during solid-phase synthesis. For minor groove modification 2F-dI-CE phosphoramidite

(Glen Research) was used. The diazirine amine tether was added via a postsynthetic modification/deprotection method as described.^[6] For oligos containing 2-F-dI modification, after deprotection with diazirine amine, 50 μ L of DBU (1,8-diazabicyclo [5.4.0] undec-7-ene) and 300 μ L formamide (heated to 55 °C) was used to remove the O6-protecting group. All oligonucleotides were purified by denaturing polyacrylamide gel electrophoresis. Concentrations of oligonucleotides were estimated by UV absorption at 260 nm.

Cross-linking Reactions and Analysis. For all cross-linking reactions, 10 mM Tris-HCl [pH 7.4] and 100 mM NaCl was used as the buffer. Typically, the protein (0.3 nmol) and modified DNA oligos (0.9 nmol) were incubated at 4 °C for 16 h. After incubation the samples were irradiated with mercury vapor lamp (Ace Glass Incorporated, Vineland, New Jersey, 450 W power supply; lights below 300 nm were cut off with a filter) for 10 min. Coomassie Blue stained SDS-PAGE was used to analyze the cross-linked product.

Melting Temperature (T_m) Measurement.^[10] Melting temperatures of normal and modified oligonucleotides were measured by using MicroCal VP Differential Scanning Calorimeter (DSC) (Northampton, MA) with 5 μ M concentration of DNA in 10 mM Tris-HCl [pH 7.4] and 100 mM NaCl buffer. Samples were scanned over the temperature range of 20-75 °C at a rate of 90 K/h. The DSC curves were adjusted from the reference buffer and T_m values were determined directly from the curves.

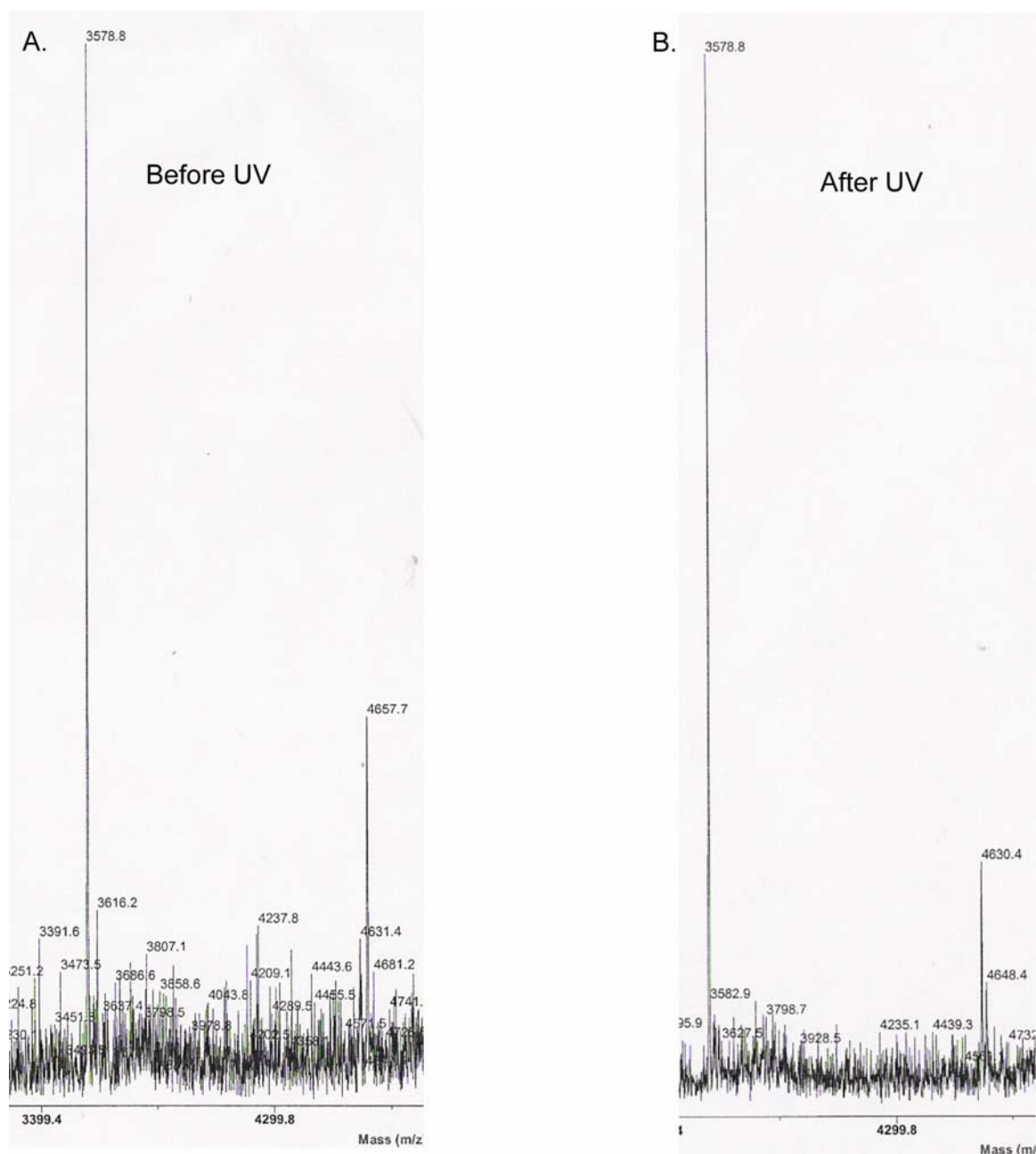


Figure S4. MALDI-TOF MS spectra of a diazirine-containing oligonucleotide before (Figure S4A) and after UV irradiation (Figure S4B). The ssDNA-1 probe containing a diazirine was UV irradiated and the mass difference (m/z) (4657.7-4630.4) of the oligonucleotide before and after irradiation is ~ 28 , which is consistent with the loss of a dinitrogen and insertion of carbene into \exists or (C-H bond to yield an olefin or cyclopropane as described previously for aliphatic diazirines.^[1] The mass difference (m/z) (4657.7-4648.4) of ~ 10 is the insertion of water molecule into the carbene. The peak at mass (m/z) 3578.8 is the mass of an oligonucleotide used as an internal standard.

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